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Chronic Copper gBAM for Fish

Investigating possibilities and limitations of a generalised bioavailability model (gBAM) for predicting chronic copper toxicity to freshwater fish

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Karel De Schamphelaere

Ghent University (UGent)
Laboratory of Environmental Toxicology and Aquatic Ecology
Environmental Toxicology Unit (GhEnToxLab)

E Karel.Deschamphelaere@UGent.be
T +32 9 264 89 19 (secretary) or +32 9 264 37 64 (direct line)

www.ugent.be
http://www.ecotox.ugent.be/

UNIVERSITEIT GENT

GhEnToxLab
Environmental Toxicology

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LIST OF ABBREVIATIONS

gBAM	Generalised bioavailability model
BLM	Biotic Ligand Model
ELS	Early life stage (test)
ECx	Effective concentration resulting in x% effect
LCx	Lethal concentration resulting in x% mortality
DOC	Dissolved organic carbon
EU-RA	European Union Risk Assessment
WHAM	Windermere Humic Aqueous Model

EXECUTIVE SUMMARY

Risk assessment (RA) of copper (Cu) in the European Union (EU) uses biotic ligand models (BLM) to account for effects of bioavailability modifying factors such as dissolved organic carbon (DOC), pH, Ca, Mg, and Na. The chronic Cu-BLM for fish that is still being used was, however, not developed *de novo* on the basis of chronic toxicity data for fish and uses the >20 year-old WHAM-ModelV to calculate equilibrium speciation of Cu. Currently, many more chronic copper toxicity data for fish are available as well as an updated and more user-friendly speciation modelling software, i.e. WHAM7. In addition, a novel type of Cu bioavailability model, which combines a log-linear pH-effect on free Cu^{2+} ion toxicity with conventional BLM-type parameters for protective effects of major cations, has been shown to be a better model than the classic BLM for chronic *Cu* toxicity to *D. magna*. Therefore, the aim of this study was to evaluate if a WHAM7-based chronic Cu-gBAM for fish could be developed from the available chronic toxicity data as a valuable alternative for the currently used chronic Cu-BLM for fish.

In a first phase, we analysed all available chronic datasets and tried to develop *de novo* gBAMs. In other words, we attempted to estimate model parameters for each dataset, fish species, life-stage, endpoint and exposure time separately. Across a range of studies with larval fish (fathead minnow and rainbow trout), we found that, in general, the effect of pH on Cu^{2+} ion toxicity, as quantified by the S_{pH} parameter of the gBAM, appeared to vary relatively widely between endpoints, studies, species, the pH range considered, and the assumptions taken for its calculation. In addition, each larval toxicity testing dataset analysed had considerable uncertainties associated with it (e.g. pCO2 used for pH control, uncertain ECx estimates). Hence, it is concluded that the effect of pH on Cu^{2+} ion toxicity to larval fish, is still unresolved. Furthermore, the effects of protective cations (Ca, Mg, Na) on Cu toxicity to larval fish, as quantified by log K values, should also be used and interpreted with care, since all estimates were only based on two data-points from a single study. Taken together, it was not possible to develop a reliable *de novo* chronic Cu gBAM specifically for the larval life stage of fish.

The same kind of analyses were performed for juvenile rainbow trout data from two studies. More weight was given to the study of Cremazy et al. (2017) than that of Waiwood and Beamish (1978a,b), since LCx estimates and speciation calculations (because availability of measured DOC values) are considerably more reliable in the former. Yet, the results of both studies were qualitatively largely comparable. Based on the Cremazy et al. (2017) dataset, an almost fully parametrised chronic Cu gBAM for juvenile rainbow trout was developed and this gBAM relatively accurately predicted effects of pH, DOC, Ca, and Mg on chronic Cu toxicity at 30d-LC20 levels. The Na effect was not investigated and hence the log K parameter for Na could not be estimated. The fact that the S_{pH} parameter decreases to an important extent with exposure time is an interesting avenue for further (mechanistic) research and shows that care must be taken when extrapolating gBAMs (or any other kind of bioavailability model) across different exposure durations. Yet, overall, the performance of this *de novo* chronic Cu-gBAM was promising in terms of its predictive capacity for juvenile rainbow trout and was, on average, better than a classic BLM type model, notably in predicting the effect of pH on juvenile rainbow trout. Hence, this gBAM was taken forward in the second study phase.

In the second study phase, we wanted to explore the performance of the developed juvenile rainbow trout gBAM from a European Risk Assessment perspective. Whilst we acknowledge that differences in bioavailability modifying effects on copper toxicity may exist between different fish species, life stages, endpoints and effect levels, it may - from a regulatory point of view - be more practical to implement a single gBAM (i.e. a single set of parameters) into risk assessment. We proposed to use the juvenile rainbow trout gBAM as the “single fish bioavailability model”, since it was undoubtedly based on the dataset of the highest quality and with the widest range of chemistries investigated (among all available studies). After an important refinement to include a Na protective effect via adding a log K for Na to the gBAM (based on evidence from larval studies), we tested how well this single juvenile rainbow trout gBAM predicted toxicity as a function of water chemistry for early life stages of fish and how far model predictions deviated from observed toxicity. With our eyes on the European Risk

Assessment, we chose to perform this evaluation on the basis of LC10 and EC10 values only, since the 10% effect level is the basis for risk assessment in the EU when using chronic toxicity studies. Given that the datasets with early life stages are not ideal to draw any strong conclusions, the results of this evaluation should, however, not be overemphasized. That said, if a 2-fold error in predicting the magnitude of the effect of a toxicity modifying factor is considered acceptable from a regulatory point of view, the gBAM performs rather well in predicting the pH effect on copper toxicity to larval fathead minnow, as evidenced from data analyses on two independent studies and in two pH ranges (i.e. 5.8-7.0 and 8.0-8.7). In the low pH range (below pH 7), the gBAM predicts a small effect of pH on dissolved copper toxicity, which coincides well with the observations and conclusions drawn from a series of tests from a single lab (OSU 2016a, 2017). For rainbow trout larvae, no definite conclusion can be drawn on how accurately the gBAM predicts the pH effect for rainbow trout larvae, although there appears to be some tendency to underestimate the pH effect. Overall, too limited experimental data is available on protective effects of major cations on copper toxicity to early life stages of fish to draw any definitive conclusions on the performance of the gBAM in this respect.

In conclusion, this study successfully developed a fully parameterised *de novo* chronic Cu gBAM for juvenile rainbow trout that incorporates toxicity modifying effects of pH, DOC, Ca, Mg, and Na. The extrapolation of this model to early life stages of fish is encouraging with respect to the effect of pH, but the available data is too limited to draw any strong conclusions with regard to protective cations. Taken together, the gBAM developed in this study is the first model to integrate all available data on the chronic toxicity of copper to fish in a consistent way, although the limitations and uncertainties of the currently available data used to develop the model must be recognized.

It is recommended to continue copper bioavailability research with fish. This should most preferably include experimental work on the Na effect on chronic copper toxicity in both larval and juvenile fish and an experimental validation of the gBAM in realistic field-collected waters. However, prior to embarking on follow-up bioavailability experiments and to select

the life-stages or endpoints to be studied, it would be wise to determine the importance of each to the overall population-level outcomes (e.g. using population modelling).

1 INTRODUCTION

The current chronic copper biotic ligand model (BLM) for fish that is used in EU risk assessment (RA) is based on a >10y-old analysis (De Schamphelaere and Janssen, 2005) of two >20y-old chronic toxicity datasets (Erickson et al., 1996; Waiwood and Beamish, 1978a,b). This chronic Cu-BLM for fish was, however, not developed *de novo*. Instead, the analysis only evaluated which of three 'animal' BLMs that already existed at that time (i.e. acute fish, acute *Daphnia*, and chronic *Daphnia*) performed best in explaining bioavailability effects and reducing water-chemistry-related variability in effective concentrations for *growth rate* endpoints. *Growth rate* was the only endpoint considered, because it was the most sensitive in both datasets. The acute *Daphnia* BLM outperformed the two other BLMs, and was therefore selected to become the chronic copper BLM for fish for EU risk assessment applications (De Schamphelaere and Janssen, 2005).

Although this chronic copper BLM is still used in EU-RA today, various additional chronic copper toxicity datasets with fish have come available (OSU, 2016a,b, 2017; Cremazy et al., 2017). In addition, it has been shown that a novel type of copper bioavailability model, the generalised Bioavailability Model (gBAM), performed better in predicting chronic copper toxicity to *Daphnia magna* over a wider range of chemical media compositions than the chronic *D. magna* BLM (Van Regenmortel et al., 2015). The gBAM combines a log-linear pH-effect on free Cu^{2+} ion toxicity with conventional BLM-type parameters for protective effects of major cations. For *D.* magna, it provides a better prediction of the effect of pH on Cu^{2+} ion toxicity than the chronic *Daphnia* Cu-BLM and requires two parameters less to be calibrated (Van Regenmortel et al., 2015).

Finally, geochemical equilibrium speciation modelling of metals, including for copper, in the presence of dissolved organic carbon (DOC) has also advanced over the past decade. Currently, the use of WHAM-Model7 is gaining increased interest in the scientific community,

at the dispense of the >20y-old WHAM-Model5, which is currently still the speciation model underlying all BLMs used in the EU-RA. Van Regenmortel (2017) recently showed that switching metal bioavailability models for copper from WHAM5 to WHAM7 maintains similar predictive capacities of metal toxicity. WHAM7 software is much more flexible and user-friendly to work with than WHAM5-software.

Given all of the above, the aim of this study was to evaluate if a WHAM7-based gBAM could be a valuable alternative for the currently used chronic copper BLM for fish. To this end we analysed both the older and the newly reported datasets. Rather than testing a single fixed *a priori* set of model parameters on all datasets (as we did in De Schamphelaere and Janssen, 2005), we tried – in a first phase - to develop *de novo* gBAMs. In other words, to the extent that the nature of the datasets allowed we attempted to estimate model parameters for each dataset, species, life-stage, endpoint and exposure time separately. By doing so, we avoided taking the *a priori* assumption that a single set of gBAM parameters can accurately capture bioavailability relationships across the entire biological landscape. This approach would also allow comparison across species, life-stages, endpoints and exposure time and thus identify factors that could drive bioavailability relationships. In a second phase, we also tested how well a single set of gBAM parameters (i.e. a single gBAM), developed based on a the Cremazy et al. (2017) data, performed in terms of toxicity predictions to other species, endpoints and life-stages.

2 MATERIAL AND METHODS

2.1 Overview of datasets used

Table 1 provides an overview of the “chronic” studies used in the current project.

Table 1. Overview of chronic toxicity studies with fish used in this project

Study	Species / test regime	Life stage(s) / acclimation to test media	Endpoints - Duration	Factors tested	Remarks
Erickson et al., 1996	Fathead minnow, , flow-through	larvae,<24h old, eggs 5-6d + larvae 12h acclimated to whole test media,	Survival, “growth” (dry weight), growth rate	pH 6.6-8.7, Ca, Mg, Na	Measured Cu^{2+} activity available

		including pH	7 days		
OSU, 2016a, 2017	Fathead minnow, flow-through	larvae, <24h old, eggs 4-5 days + larvae 12h acclimated	Survival, "growth" (dry weight) 7 days	pH 6-7	pH 6 repeated twice, Cu contamination in controls of some tests, few partial effects in most tests, equilibrium Cu^{2+}-DOC binding confirmed
OSU, 2016b	Rainbow trout, flow-through	Fertilised embryo (19 dpf) to swim-up larvae Gradual acclimation of embryos to media, including pH, for 5 days	Survival, hatching, larval weight 52d (10d embryo+12d alevin+30d swim-up)	pH 6-7	Hardly any effects observed at any Cu concentration, therefore some uncertain LCx and ECx estimates, equilibrium Cu^{2+}-DOC binding confirmed
Cremazy et al., 2017	Rainbow trout, flow-through	juvenile 5-10g, 1day pre-acclimation (pH series only)	Survival, weight 30d	pH 5.1-8.6, Ca, Mg	No growth effects at all, pH series already reported in Ng et al. (2010), w/ adjusted DOC estimate
Waiwood and Beamish, 1978a,b	Rainbow trout, flow-through	Juvenile, 6-10g No pre-acclimation to conditions (pre-culture pH 8, hardness 365 mg CaCO3/L)	Survival, growth rate 30d	pH 6-8, hardness	LC20s estimated "by eye" on probit paper Most LCx extrapolated, ECx(growth) quasi impossible to estimate Only 2 Cu conc. per medium. Hardness likely correlated with assumed DOC concentration (since hardness modification via source water dilution. DOC not measured

Because of a lack of true chronic bioavailability studies with fish (i.e. test duration significant portion of life-cycle), we considered early life stage tests and growth experiments with larval and juvenile fish as equivalent to chronic. This included short-term chronic studies on *P. promelas* following USEPA methods, which are a good proxy for longer term tests (Norberg & Mount, 1985). We only considered studies which had at least one endpoint that is considered relevant for chronic toxicity in EU-RA. Similar to what we did previously (De Schamphelaere and Janssen, 2005), we only evaluated studies in which copper toxicity was tested in at least two media with different chemistries. More chronic fish toxicity studies are certainly available, but bioavailability relationships can only be reliably inferred from comparative toxicity tests run in the same laboratory. Otherwise it is not possible to

differentiate a bioavailability effect of any given water chemistry variable from mere inter-laboratory variability. A total of 5 studies was retained for the current project (Table 1). We considered all reported exposure durations, but required that organisms were fed during the tests.

2.2 Dose response analysis

For all but one study, LCx and ECx values were used as reported in the original studies. However, in order to enable a larger set of endpoints and effect levels to be considered for comparative purposes, raw data from the Erickson et al. (1996) study (obtained via personal communication with RJ Erickson in 2005) were used for calculating additional LCx values for *survival* and ECx values for *dry weight*. These endpoints had not been calculated or considered in our previous chronic fish BLM work (De Schamphelaere and Janssen, 2005). LCx and ECx values were calculated with the 2-parameter log-logistic model (Equation 1), using the *drc package* in R software.

Equation 1. The two parameter log-logistic model (*llogistic2*). The parameters *b* and *e* are the slope of the dose-response curve and the natural logarithm of the median effective concentration (EC50), respectively

$$f(x) = \frac{1}{\left(1 + \exp\left(b * \left(\log(x) - e\right)\right)\right)}$$

2.3 General approach and estimating gBAM parameters

As mentioned in the introduction, our starting point was that different species, different life-stages, different endpoints, and different effect levels could respond differently to copper-bioavailability modifying parameters like pH, Ca, Mg and Na. For the analyses below, we grouped the studies by life-stage (and not by species as is often done) to emphasize that early life stages (embryo, alevin & swim-up larvae) can have vastly different physiology than juvenile fish, including presence/absence of active feeding and ion-regulatory processes,

which could result in different toxicity mechanisms and pathways between life stages and this in differences in bioavailability relationships (e.g. if a water chemistry variable affects key physiological processes that have been implicated in copper's toxic mode(s) of action.

This resulted in two groups of datasets (grouped by life stage):

(1) Erickson et al. (1996) and OSU (2016a,b, 2017): Early Life Stage (ELS) tests, i.e. 7d-larval fathead minnow tests and a 52-day ELS test with rainbow trout (embryo+alevin+swim-up larvae, continuous).

(2) Cremazy et al. (2017) and Waiwood and Beamish (1978a,b): 30d-juvenile rainbow trout tests

First, each dataset will be analysed and interpreted separately. In addition, a general synthesis, comparing studies, endpoints and effect levels will be presented for early life stages (3 studies) and juveniles (2 studies). Finally, a comparison between life stages will be presented and a general conclusion and remaining uncertainties and recommendations will be formulated.

The analysis of every dataset begins with calculating LCx and ECx values expressed as Cu^{2+} ion activity and to evaluate how toxicity on free Cu^{2+} ion activity basis changes as a function of water chemistry variables. This allows to discriminate between speciation effects of water chemistry (i.e., complexation with DOC and inorganic ligands) and effects on the organism (e.g. competitive uptake, physiological effects). The next section explains how speciation calculations were performed.

We will do this endpoint-by-endpoint, i.e. we do not a priori assume that model parameters are invariant among endpoints. Relationships between LCx and ECx values expressed as free Cu^{2+} activity and water chemistry are used for estimation the parameters of the gBAM model (equation 2). Where possible the 4 parameters of the gBAM will be estimated from univariate test series (i.e. univariate in the sense that only one variable occurring in the

equation is modified among a set of test media at once). The general form of the gBAM is given in equation 2 (Van Regenmortel et al., 2015).

Equation 2. The generalized bioavailability model (gBAM), predicting ECx as free Cu2+ ion activity (mol/L) as a function of the intrinsic sensitivity, Q_x (dimensionless), the pH slope parameter, SpH (1/pH unit), pH, BLM-type cation competition parameters K_{CaBL}, K_{MgBL} and K_{NaBL}, (L/mol) and free ion activities of Ca2+, Mg2+ and Na+ (mol/L).

$$ECx_{Cu2+} = 10^{Qx - S_{pH} \times pH} \times \left\{1 + K_{CaBL}(Ca^{2+}) + K_{MgBL}(Mg^{2+}) + K_{NaBL}(Na^{+})\right\}$$

The gBAM combines (assumed) competition between Cu^{2+} and competing ions (Ca^{2+}, Mg^{2+} and Na^{+}) for an (assumed) single unidentate biotic ligand site with an empirical log-linear pH effect on the toxicity expressed as free Cu^{2+} ion activity. The effect of pH is assumed not to interact with the effect of the competing ions (hence, the multiplication in equation 2). The empirical log-linear pH effect is assumed to integrate in a non-mechanistic manner all possible mechanisms by which pH can affect the toxicity expressed as the free Cu^{2+} ion activity, such as potential toxicity contribution of complexes like $CuOH^{+}$ and $CuCO_3$, competition between H^{+} and Cu^{2+}, and differences between bulk pH and gill-microenvironment pH. The S_{pH} can be estimated as the slope of the negative linear regression between log(ECx as Cu2+) and pH (Van Regenmortel et al., 2015), whereas the competition parameters can be estimated from the positive slope of the linear regression between ECx (as Cu2+) and the activity of the competitive cation.

2.4 Speciation calculations

All equilibrium speciation calculations were performed in WHAM7 software (NERC, 2011; Tipping et al., 2011), using default settings and default thermodynamic databases of binding constants (except for copper inorganic complexing), with following assumptions:

(1) NIST constants were used for inorganic complexing of copper

(2) DOM (all from natural sources in all considered studies) was assumed to contain 50% carbon on weight basis and was assumed to consist of 65% active fulvic acid; hence the fulvic acid (FA) input in the speciation model was as follows: FA = 1.3 x DOC (Bryan et al., 2002).

(3) Activity of Fe^{3+} was assumed to be under control of colloidal $Fe(OH)_3$, and was calculated from pH, following the default function in WHAM7 software

Furthermore, we assumed equilibrium copper speciation was attained in all exposure media. This is experimentally supported by data for the OSU (2016a,b, 2017) studies, but not for Cremazy et al. (2017) and Waiwood and Beamish (1978a,b). This is not an issue for Erickson et al. (1996) as Cu^{2+} activity was measured, enabling us to work directly with measured Cu^{2+} activities. According to their method description, these measurements were performed in a similar manner as our own measurements used to develop chronic algae bioavailability models for copper and for calibrating WHAM to measured copper speciation (De Schamphelaere et al., 2002; 2003).

3 RESULTS AND DISCUSSION

3.1 Bioavailability effects on copper toxicity to early life stages

Three datasets are considered, (1) one in which the effects of pH over a wide range (6.6-8.7) and major cations (Ca, Mg, and Na) on copper toxicity were investigated in fathead minnow (Erickson et al., 1996), (2) another one in which the effects of pH in the low pH range (5.8-7.0) were investigated in fathead minnow (OSU, 2016a; OSU 2017), and (3) a final one in which the effect of pH (5.8-7.0) on rainbow trout early life stage was investigated (OSU, 2016b).

3.1.1 Larval fathead minnow (Erickson et al., 1996)

Erickson et al. (1996) reported results of a total of twenty-four 7-day larval toxicity tests (ELS tests), spread over 6 test series. Within each test series all tests were conducted simultaneously (as mentioned explicitly in their materials and methods section). Following

the same reasoning as De Schamphelaere and Janssen (2006), results of tests in which DOC, suspended solids or alkalinity (high K, unrealistic K/Na ratio) were modified were not considered useful for estimating bioavailability model parameters. In addition, the test series F6, in which alkalinity was modified independent of pH, were also excluded in the current analysis, because they did not vary in any of the gBAM variables (equation 2) and because the raw data on measured Cu^{2+} ion activity for this test series were not found back. Thus, we only focus on test series F1 (effects of major cations) and F2 (effect of pH).

In our previous BLM work with this dataset (De Schamphelaere and Janssen, 2005), we only focused on the endpoint growth rate, which we calculated ourselves from initial (100 µg dry wt/fish) and final dry weights, and used estimated 7d-$EC60_{gr}$ as the effects estimator, since the 60% effect level provided one more non-extrapolated effect concentration estimate than the 50% effect level did (i.e. for test F1-A). Growth rate is, in our opinion, a more relevant endpoint than weight (at end of test), because effects on it can be more directly incorporated into population models. However, in the present study, with the aim of enabling optimal comparison between studies, we also calculated 7d-LCx (endpoint: survival) and 7d-ECx values (endpoint: dry weight/fish), with x=10,20,50, and 60. All estimated LCx and ECx values are provided in Supplementary Information (section 7.1, Table S1 and S2).

Before going into the gBAM analysis, a few general observations are important to note:

- For the endpoints *dry weight* and *growth rate*, no reliable ECx values could be calculated for test F2-B (elevated Ca), since close to 100% effect was already observed at the lowest tested Cu^{2+} activity (i.e. 27 nM). This a priori disables estimating a value for log K_{CaBL} from this dataset.
- For the endpoint *dry weight*, all EC10, all EC20 and 3 out of 7 EC50 values were extrapolated below the lowest tested Cu^{2+} activity. In contrast, all 7d-$EC60_{dw}$ are within the tested Cu^{2+} activity range. As a consequence, the 60% effect level is the only effect level to infer reliable bioavailability relationships (and estimate gBAM

parameters) for the endpoint *dry weight*. It is also the most appropriate effect level to compare findings between endpoints.

- At the 60% effect level, it is very clear that the endpoints *dry weight* and *growth rate* show very similar sensitivity (EC60 ratio between 0.8 and 1.4, geometric mean 1.0, n=7). It is also very clear that *dry weight* (and thus also *growth rate*) is a more sensitive endpoint than *survival*, ranging from 1.9 to 6.5-fold for 6 of 7 tests (Figure 1). The only exception is test F2-D, which had the lowest pH (i.e. pH 6.6), for which similar sensitivity between endpoints was observed (Figure 1).
- Since tests F1-A and F2-A are tests from different test series (i.e. not conducted simultaneously) that have quasi-identical chemical composition (certainly in terms of the four gBAM variables, equation 2), comparison of results between those two tests allows an estimate of between-test variability of copper toxicity. At the 60% effect level, fathead minnow larvae appeared 1.8, 2.7 and 2.0 fold more sensitive in F1-A than in F2-A for the endpoints *survival*, *dry weight* and *growth rate*, respectively. For the endpoint *survival*, the sensitivity difference tends to be higher at lower effect levels, i.e. up to 3.8-fold at the 7d-LC10 level. To exclude any possible interference of between-test-series-variability with estimation of gBAM parameters, we decided to not combine results from the two test series for such estimations. Thus, S_{pH} was estimated from series F2 alone, while log K_{CaBL}, log K_{MgBL}, and log K_{NaBL} were estimated from series F1.

In what follows, we used the estimated LCx and ECx values for estimating the gBAM parameters and compare across endpoints and effect levels as appropriate.

3.1.1.1. pH effect for larval fathead minnow (Erickson et al., 1996)

Figure 1 shows LC60 and EC60 values (expressed as Cu^{2+} activity) as a function of pH. This figure again shows that dry weight and growth rate are more sensitive endpoints than

survival, except at the lowest pH, where sensitivity appears quasi-identical (the point for survival hidden behind those for dry weight and growth rate).

Consistent with the gBAM formulation, a log-linear relation is a very good descriptor between log(ECx) and pH for endpoints *dry weight* and *growth rate*, explaining >90% of the almost one order of magnitude (10-fold) sensitivity difference between pH 6.6 and pH 8.7. In contrast, it is a very poor descriptor for the endpoint *survival*, for which the relation appears inverse U-shaped, in line with what Erickson et al. (1996) reported for 96h-LC50s (see their Figure 1). Thus, in this test series, a gBAM-consistent pH effect does not seem to explain the 0.7 log-unit toxicity variation (5-fold variation) across the investigated pH range for the *survival* endpoint. Erickson et al. (1996) were not able to provide a sound explanation for this effect either, but suggested that detailed study of copper binding (uptake) to (in) gills and the pH of the gill micro-environment could help to explain the observations. Yet, it should be acknowledged that larval fish do not have fully developed gills and that, for instance, the dominant site of Na uptake (a likely target for Cu toxicity) shifts from skin to gills during larval development (Zimmer et al., 2014; 2017).

Worth noting is that in two of the four tests of series F2 (pH 6.6 and pH 7.3) the target pH was achieved via addition of CO_2 gas. It has been reported that increased $pC0_2$ of exposure water, which is an uncommon situation in freshwater bodies in the field, can increase metal toxicity, most likely because elevated pCO_2 immediately depresses the pH of the internal body fluids of the animal (“respiratory acidosis”) (Esbaugh et al., 2013). If we only consider those two tests in which no CO_2 has been added (i.e., pH 8.0 and 8.7), a pH effect that is consistent with the gBAM is also observed for survival (Figure 1). Yet, this is only supported by two data-points and it remains unexplained then as to why increased pCO_2 would only affect copper-induced effects on survival and not on growth. On the other hand, if pCO_2 was not present at levels sufficient to affect copper toxicity, it could be hypothesized that the different pH effect on copper toxicity between survival and growth endpoints, is a result of

different toxicity pathways leading to effects on survival vs. growth. The latter is supported by evidence that Cu induced mortality in fish is associated with Na loss (Grosell et al., 2002), and by some limited evidence that growth effects of Cu correlate with tissue and whole body copper burden (Marr et al., 1996; Hanssen et al., 2002). Research in which both Na uptake, Na loss, Cu accumulation, survival and growth are monitored during Cu exposure at a range of pH levels (not controlled using pCO_2) would shed more light on this hypothesis.

Estimated values of S_{pH} are provided in Table 2 for various effect levels and endpoints. When considering all 4 pH levels (6.6-8.7), S_{pH} values for growth rate and dry weight are similar at 0.382 and 0.418, respectively. S_{pH} values for survival are low (<0.15), and even virtually zero considering the low proportion of variability explained by the linear relation (<20%). When considering only the two data points without CO_2 used for pH control (pH range 8.0-8.7), S_{pH} values would be between 0.714 and 1.030 for *survival* (depending on the effect level, 1.030 at LC60), and 0.810 for growth rate and 0.466 for dry weight (both at LC60). All these values are within 2-fold, but the 'value' of this comparison should not be overemphasized, given it is only based on two tests within a narrow pH range (8-8.7).

Table 2. Estimates of the S_{pH} parameter of the gBAM for various endpoints and effect levels of larval fathead minnow and rainbow trout

	Fathead minnow (Erickson et al., 1996, all tests, pH6.6-8.7, n=4, 7 days)	Fathead minnow (Erickson et al., 1996, w/o increased pCO2 tests, pH 8-8.7, n=2, 7 days)	Fathead minnow (OSU, 2016a, scenario 1, true pH effect on dissolved Cu, pH 5.8-7.0, n=3, 7 days)	Fathead minnow (OSU, 2016a, scenario 2, no pH effect on dissolved Cu, pH 5.8-7.0, n=3, 7days)	Rainbow trout OSU, 2016b, pH 6-7, n=2, 52 days)
Survival, LC10	ND (extrapolated LC10)	0.714	0.200	0.703	0
Survival LC20	ND (extrapolated LC10)	0.812	ND	ND	ND
Survival, LC50	(0.137) r2=0.2	0.981	ND	ND	ND
Survival LC60	(0.133) r2=0.17	1.030	ND	ND	ND
Dry weight, EC10	ND (extrapolated LC10)	ND	(0.154) low r^2	0.692	ND
Dry weight EC20	ND (extrapolated LC10)	ND	(0.094) low r^2	0.668	ND
Dry weight, EC60	0.418	0.810	ND	ND	ND
Growth rate EC60	0.382	0.466	ND	ND	ND

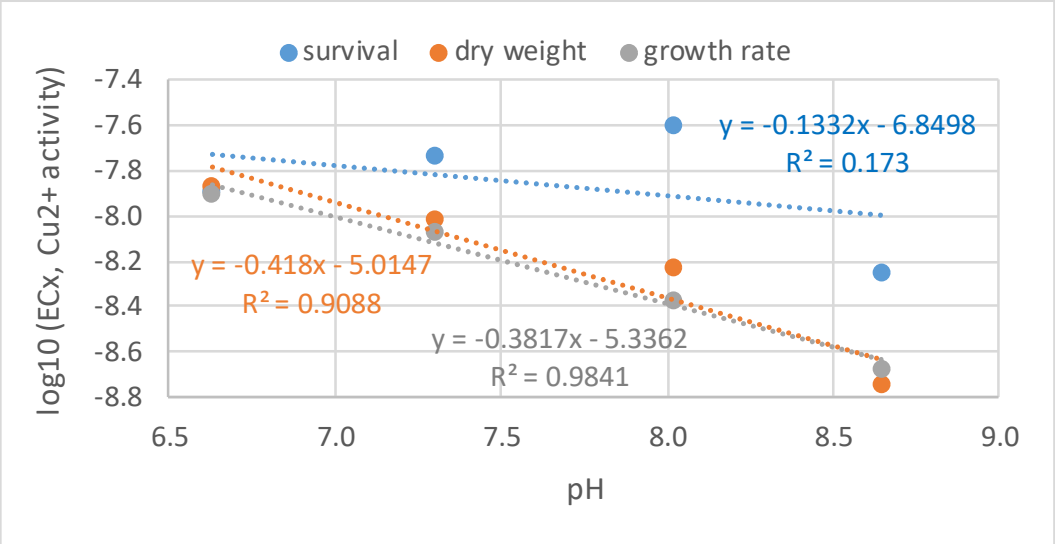

Figure 1. Effect of pH on copper toxicity (60% effect level expressed as free Cu2+ ion activity) to different endpoints of a 7-day tests with larval fathead minnow (data from Erickson et al., 1996).

3.1.1.2 Effect of major cations in larval fathead minnow (Erickson et al., 1996)

Protective effects of Ca^{2+}, Mg^{2+}, and Na^{+} ions on toxicity expressed as Cu^{2+} ion activity were reported. Estimated values of log K_{CaBL}, log K_{MgBL}, and log K_{NaBL} for various endpoints and effect levels are reported in Table 3.

Table 3. Estimates of log K_{CaBL}, log K_{MgBL}, and log K_{NaBL} parameters of the gBAM for various endpoints and effect levels in larval fathead minnow

	log K_{CaBL}	log K_{MgBL}	log K_{NaBL}
Survival, LC10	3.2	3.6	3.3
Survival LC20	3.3	3.2	3.1
Survival, LC50	3.4	2.2	2.8
Survival LC60	3.5	ND (negative intercept)	2.7
Survival (mean of LC10, LC20, LC50)	3.3	3.0	3.1
Dry weight, EC60	ND (no EC60 at high Ca)	2.9	3.4
Growth rate EC60	ND (no EC60 at high Ca)	3.1	3.5

It is noted that all estimates are based on linear regression between two points only (basis medium, F1-A, and medium with added major cation, F1-B to F1-D). Some log K values could not be determined, due to lack of an ECx value (high Ca, F1-B test) or due to negative intercepts of the linear regression. It is also noted that the log K_{MgBL} for *survival* is variable and uncertain, because the dose response data in the high Mg medium (F1-C) only showed one partial response, resulting in a steep dose-response curve and in the large variation of log K_{MgBL} (1.9 units) among the various effect levels. This variation was much smaller for log K_{CaBL} (mean value for *survival* of 3.3) and log K_{NaBL} (mean value for *survival* of 3.1). Interestingly, for *survival,* the mean values of log K are quite similar for all cations. Log K_{MgBL} values appear similar between *survival*, *growth rate* and *dry weight*. In contrast, as reflected in the log K_{NaBL} values, there appears to be trend of a somewhat stronger Na protective effect from high to low effect levels for *survival* and also a stronger Na effect on *dry weight* than on *survival*. If this would be confirmed in an experiment with a wider range and higher

number of Na concentrations, this would support the idea of the existence of possibly two different toxicity pathways leading to survival and growth effects (as mentioned above), with Na playing a stronger, possibly physiological, role in the growth-effect pathway than in the survival-effect pathway.

3.1.2 Larval fathead minnow (OSU, 2016a, 2017)

Oregon State University performed 7d ELS copper toxicity tests with fathead minnow larvae at pH 6 (twice, #1018 and #1023), 6.5 (#1019) and 7 (#1020) (OSU, 2016a), which were performed sequentially over a period of four weeks (OSU, personal communication), and another repeated test at pH 6 (OSU, 2017). The latter test (#1124) was conducted as a follow-up study to confirm the results of tests #1018 and #1023, both of which suffered from some Cu contamination in the controls The studies considered the endpoints *survival*, *dry weight*/*fish* (although they call this, like many other authors, 'growth', we prefer to call it *dry weight* for consistency reasons and to discriminate from *growth rate*) and *total biomass*. We did not consider the latter endpoint as it is does not add extra information, since it equals *survival* x *dry weight*, and one would even lose information on possible mechanisms of toxicity or bioavailability when only the *total biomass* endpoint would be considered. Thus, even if this endpoint would be the most sensitive one, it is appropriate not to consider it here.

Before going into the gBAM modelling, following observations are of importance:

- The repeated tests provide some insight in the between-test variability under otherwise very similar conditions. In the first pH 6 test (#1018) the LC10 and $EC10_{dw}$ were 10.1 µg/L and 8.6 µg/L, respectively, with 25% *mortality* and 35% reduction of *dry weight* at the highest test concentration of 12.8 µg/L (both significant effects). In contrast, no significant effects (0% mortality and 4% reduction of *dry weight*) were observed at a comparable concentration of 11.9 µg/L in the second pH 6 test (#1023). It thus appears that copper toxicity was higher than in the first test, but because no higher concentrations were tested in both the first and the second pH 6

test, it is impossible to quantify the difference. In the third pH 6 test (#1124, OSU, 2017), the LC10 and $EC10_{dw}$ were 18.9 µg/L and 25.2 µg/L, respectively, with 0% mortality and 0% growth reduction at 11.2 µg/L and 12% mortality and 5% growth reduction at 23.2 µg/L (all effects not significant). The third pH 6 test (#1124) was conducted considerably separated in time relative to #1018 and #1023, but on the other hand, a better dose-response curve could be fitted, and it did not have contamination in the control treatments. This third test (#1124) appears to show a 1.9 - 2.9 fold lower sensitivity than the first pH 6 test (#1018). Interestingly, this is similar to the between-test variation described above based on the study of Erickson et al. (1996). In contrast, the authors themselves (OSU, 2017) consider the third test as a succeeded confirmation of the first pH 6 test result, based on overlapping confidence intervals. However, if that rationale would be consistently followed it would be impossible to detect any effect of pH on (dissolved) copper toxicity (in this study) that is smaller than 2-3 fold, unless all pH levels were investigated simultaneously (or if much more pH levels would be repeatedly tested). As we believe it is reasonable to assume that the likelihood of increased between-test-sensitivity variation is higher when tests are more separated in time, we decided to restrict our modelling effort to tests #1018, #1019 and #1020, which were all conducted within a 3-week period, while ignoring some additional limitations of the OSU (2016a) study, as described below.

- Two (#1018, #1019) of the three originally planned tests (#1018, #1019 and #1020) only had a single copper treatment that displayed a ‘partial’ effect (sufficiently different from 0% or 100% effect). In such a case, the estimated effective concentrations are considerably uncertain, as the slope of the dose-response curve are difficult to estimate, which the authors of the OSU (2006) report in fact also mention. Hence, even if the dose-response-fitting program returns LCx or ECx values, such values should be processed carefully when taken forward in estimating parameters of bioavailability models, like the gBAM.

- Average dissolved copper concentrations in controls of the three original tests (#1018, #1019 and #1020) ranged between 3.6 and 5 µg/L, which could be considered to have already caused some toxicity in the controls and thus to have influenced the estimated LC10 and EC10 values. However, in all these tests, the endpoints survival and dry weight showed a more or less constant response over an at least 2-3 fold range of copper before they showed some significant decrease (see Figure 2 – dose response curves). Therefore, despite this arguably non-optimal situation, we believe the relatively high background Cu in the controls is unlikely to have caused any important influence on the estimated LC10 or EC10 values.
- Relatively similar toxicity between *survival* and *dry weight* is observed. The dose-response curves suggest a slightly higher sensitivity for *dry weight*, but comparison between LC10 with $EC10_{dw}$ values suggests relatively small differences ($EC10_{dw}$/LC10 ratio range 0.9-1.4, geometric mean: 1.1, n=3). This corroborates with the similar effect size on growth vs. survival reported for the lowest pH tested by Erickson et al. (1996), but not with the other pH levels they tested (see 3.1.1.1).

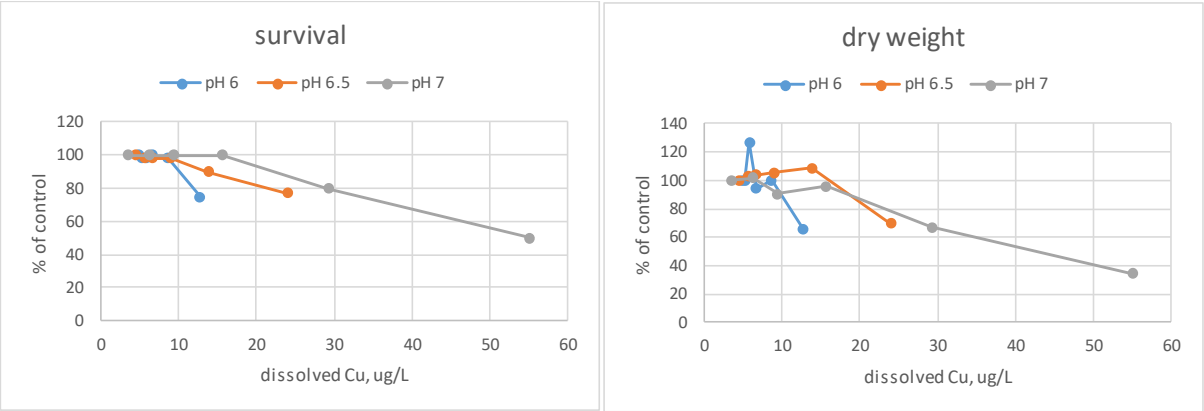

Figure 2. Effect of pH on copper toxicity as shown by dose response data for survival and dry weight (data from OSU, 2016a). Only data from tests #1018, #1019, and #1020 are shown, since those were conducted sequentially over a period of 3 weeks and thus between-test variability can be assumed small (see text).

Given the uncertainty around two out of three estimated LC10 and EC10 values, and taking into account the between-test variability suggested by comparison between #1018 and #1023, even within the short period in which all tests were performed, we decided to take this into account in our gBAM analysis, by considering two scenarios

1. Scenario 1: the reported effects of pH on dissolved copper toxicity are small, but real. This does not seem implausible when considering the plotted concentration response data (Figure 2). This scenario also assumes zero between-test-sensitivity variability among tests #1018 to #1020 (i.e., simultaneous testing). The actual estimated values of LC10, EC10 and EC20 at each pH level were used for speciation calculations.
2. Scenario 2: Considering all data, including those of repeated tests #1023 and OSU (2017), the effects of pH on dissolved Cu toxicity are considered not significant (within tested pH range); all variation is within 'normal' variation of repeated toxicity tests. This corroborates with the conclusion of OSU (2017), which stated, combining results of all conducted tests (OSU, 2016a, 2017), that there is "no significant effect of pH on [dissolved] copper toxicity on fathead minnow ELS". In this scenario, we used a single average LC10, EC10 and EC20 value (average across the three pH levels, tests #1018 to #1020) for speciation calculations.

In both scenario's, we calculated free Cu^{2+} ion activities at the LC10, EC10 and EC20 levels using the chemistry as reported in OSU (2016). We assumed equilibrium of copper speciation, which is supported by speciation measurements (CIMM, 2016) and *Ceriopdahnia dubia* copper toxicity tests (OSU, 2016a) with different equilibration times (CIMM, 2016; OSU, 2016a). The measured pH value in treatments close to the LC10, EC10 and EC20 were used (i.e. treatment 8 µg/L in table 3-6, treatments 12.5 and 25 µg/L in table 3-8, and treatments 17.5 and 35 µg/L in Table 3-9 in OSU, 2016a). For other chemistry variables, the mean of the measurements in control and highest Cu treatment was used (their tables 3-1, 3-3, 3-4).

The relation between free Cu^{2+} ion toxicity and pH is depicted in Figure 3. Estimates of SpH are provide in Table 3. Under scenario 1, the overall variation in toxicity is only 0.3 log-units (i.e. 2-fold) and only for the LC10 value a relatively good correlation is observed. Depending on the endpoint and effect level considered, estimated SpH values vary between 0.094 and

0.200. Under scenario 2, the effect of pH on Cu^{2+} ion toxicity is estimated to be stronger, i.e. almost 10-fold variation between pH 5.8 and 7.0. A very strong log-linear relation between pH and copper toxicity is found, consistent with the gBAM, although this merely reflects the log-linear relation of Cu^{2+} ion activity with pH that results from speciation (since dissolved Cu was held constant in this scenario). S_{pH} values are nearly identical among endpoints and effect levels, with a mean S_{pH} = 0.688. Thus, the two scenarios yield quite different conclusions regarding the pH effect on Cu^{2+} ion toxicity. Considering both scenarios as the two extremes, the actual lower and upper boundary of the true SpH values (for the pH range 5.8-7.0) could be considered as ~0.2 to 0.7 for *survival* and 0 to 0.7 for *dry weight*.

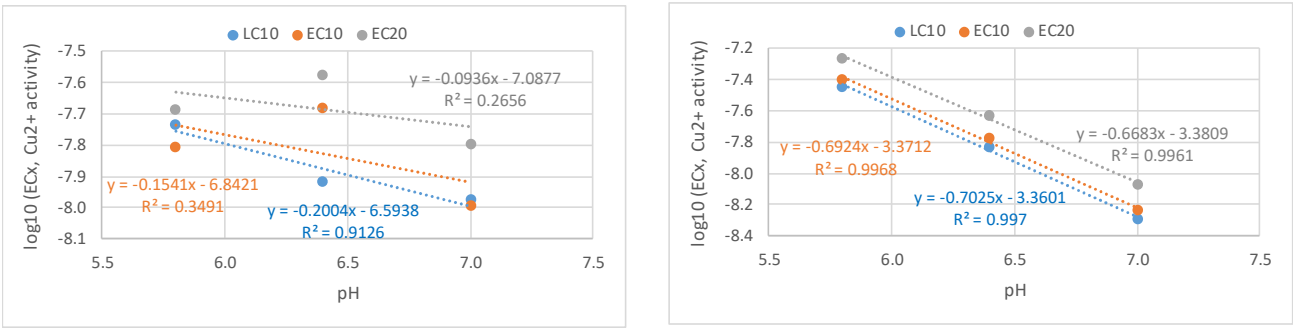

Figure 3. Effect of pH on Cu^{2+} ion toxicity on survival and dry weight under scenario 1 (left) and scenario 2 (right), as explained in the text (OSU, 2016a, 2017 datasets).

3.1.3 Early life stage rainbow trout (OSU, 2016b)

OSU (2016b) performed early-life stage tests at two pH-levels (pH 6 and pH 7), starting from 19d-post fertilization eyed embryo's. *Embryo survival, time to hatch* and *time to swim-up* in the so-called 'alevin' stage (from day of hatch at about 10 days after test initiation until 22 days after test initiation) were monitored. No food was provided until day 22. From that day onward, the so-called 'swim-up larvae' were fed *ad libitum* and continued to be exposed for 30 more days, and *larvae survival* and a number of growth related parameters were recorded at the end of the exposure. Hence, the total exposure time was 52 days, and the exposure as larvae (alevin + swim-up stage) lasted 42 days. Because effects expressed as ECx on *time to hatch* and *time to swim-up* are not easily extrapolated to population level (*development rate* would be better) and because of reasons of comparability with the other studies considered in this report, we restricted our analysis to the endpoints *embryo survival*,

larvae survival (i.e. %survivors among hatched individuals) and *(larval) dry weight* at the end of the exposure. It should be noted though that the larvae endpoints are not identical to those reported above for fathead minnow, because the latter did not include exposure of the preceding embryo stage. It is known that prior exposure of fish embryo's may alter the metal sensitivity in the subsequent larval phase. Embryo's had been acclimated for 5 days to the test pH. Both pH levels were tested simultaneously and used the same batch of eggs.

Before starting any modelling, following observations are important to consider:

- No effects on *embryo survival* were observed up to and including the highest test concentration (i.e. 48.4 µg/L at pH 6 and 59.0 µg/L at pH 7). It is therefore impossible to estimate the bioavailability relationship between pH and Cu^{2+} ion toxicity for this endpoint.
- In the larvae stage, significant effects were only observed on *survival* at the highest test concentration (48.4 µg/L) in the low pH. This makes the 30d-LC10 (*larvae survival*) and 30d-EC10 (*larval dry weight*) estimates particularly uncertain and thus any model parameter estimations resulting from it should be interpreted with care. Significant effects.
- Based on observed effects at 48.4 µg/L in the pH 6 test; *larvae survival* (48.7% mortality) appears to be more sensitive than *larvae dry weight* (~1% reduction relative to control), but an exact quantification of the difference is not possible because of a lack of reliable LCx and ECx values.
- Based on *survival*, copper toxicity (on dissolved basis) appears to be somewhat higher at low pH (48.7% mortality at 48.6 µg/L) than at high pH (16.5% mortality at 59.0 µg/L). This is supported by the author's (uncertain) estimates of the 42d-LC10 values, i.e. 28.5 µg/L at pH 6 and 54.2 µg/L at pH 7. To our interpretation, the latter LC10 value rather appears to be a lower boundary of the true LC10 value, since mortality in the highest copper concentration (59 µg/L) is 16.5% (i.e., within ~6% of

the control mortality of 11.2%) and since an upper confidence limit could not be calculated, i.e. LC10 ≥54.2 µg/L at pH 7. This should be accounted for when trying to estimate the SpH parameter for this experiment. Conclusions on the pH effect on the endpoint *dry weight* can, in our opinion, not be drawn at all, because no significant effects (and in both cases below or close to the control dry weight) were found at the highest concentration in both pH levels.

Based on the above, we decided to only estimate an SpH value for 42d *larvae survival* based on the reported LC10 values and using the chemistry reported in the tables of the report (we actually used the same chemistry input for speciation calculations as the one used by Cremazy et al., 2017, who also tried to model these data, see their supporting information). We found LC10 values expressed as calculated free Cu^{2+} ion activity of 63 and ≥65 nmol/L at pH 6.1 and 7.03, respectively. This suggests a very limited effect of pH on copper ion toxicity to larval survival (when also exposed as embryo), with an SpH~0 (Table 2). This is in agreement with our calculations on Erickson et al. (1996) data for pH 6.6-7.3 (Table 2), although the latter dataset has the possible issue of CO_2 additions to control pH. It is also in agreement with scenario 1 of our calculations with OSU (2016a) data on FHM (also relatively small pH effect), but in disagreement with scenario 2 of the same dataset (somewhat stronger pH effect) (Table 2).

3.1.4 Conclusions, remaining uncertainties and recommendations for larval fish

In general, the effect of pH on Cu^{2+} ion toxicity for larval fish, as quantified by the S_{pH} parameter appears to vary between endpoints, studies and species, and could also depend on the pH range considered and assumptions for its calculation. Overal S_{pH} values between 0 and 1 were found (Table 2), which means that free Cu^{2+} ion toxicity could increase anywhere between 0 and 1-log-unit per unit pH increase. Given that each larval toxicity testing dataset considered here has considerable uncertainties (as described above), and given that vastly different conclusions can be drawn, depending on assumptions or

scenario's taken, it must be concluded that the effect of pH on Cu^{2+} ion toxicity to larval fish, a crucial knowledge component for gBAM development, is still unresolved and loaded with uncertainty. The most important issues in the datasets causing the uncertainties are the use of pCO2 for pH control (Erickson et al., 1996) and between-test variability and uncertain LCx and ECx estimation related to a low number of copper treatments with partial effects (OSU, 2016a,b, 2017). This could nonetheless be easily resolved by testing copper concentrations that span a wider range of effects (allowing reliable LCx and ECx calculations) and this over a wide pH range, while avoiding the use of CO_2 for pH control. Before embarking on this, it would be wise to test how the uncertainty/variability observed here would propagate into true population level effects (i.e. how important is effect on larvae survival and growth in terms of population growth and population density, via sensitivity analysis using population models). An interesting hypothesis to follow-up one is whether the different effect of pH on copper toxicity to growth vs. survival might be related to different toxicity pathways for those two endpoints.

The effects of protective cations, as quantified by log K values (Table 3), should also be interpreted and compared with care, since all estimates were only based on two data-points from a single study. That said, protective effects of Ca, Mg, and Na on Cu^{2+} ion toxicity to survival appear all relatively similar. The effect of Mg appears relatively similar across endpoints, while the protective effect of Na appears to be stronger for *dry weight* and *growth rate*. The latter may suggest different toxicity pathways leading to lethal vs. growth effects. Here too, it would be worthwhile to confirm these trends with additional testing, including a wider range of concentrations of Ca, Mg and Na.

As a final note, it is worth mentioning that Erickson et al. (1996) reported that 96h-copper toxicity (for survival) was similar between fed and non-fed larvae. Capitalizing on this statement, it could be interesting to test if bioavailability relations are similar among fed and

non-fed larvae, using the available data. If these relations are sufficiently similar, perhaps parameters derived from non-fed toxicity tests could be used in the chronic gBAM.

Finally, it can only be concluded that uncertainties related to the pH effect and the limited number of data-points available for estimating specific gBAM competition constants, currently prohibit the development of a reliable chronic Cu gBAM specifically for the larval life stage of fish. It must also be emphasized that a single set of gBAM parameters may not be sufficient to describe bioavailability effects on survival and growth endpoints.
That said and with an eye on implementation of bioavailability-normalisation of chronic fish toxicity data in risk assessment, we will further in this report test and discuss how well a gBAM developed specifically for juvenile fish (rainbow trout, section 3.2) performs for the various above-reported datasets on early life stages of fish (see section 4).

3.2 Juvenile fish (rainbow trout)

Two datasets are available. Waiwood and Beamisch (1978a,b) investigated copper effects in juvenile rainbow trout (5-10g/fish) over a 30d-exposure period at various pH and hardness on *survival*, *growth rate*, *feeding rate*, *gross conversion efficiency, critical swimming performance* and *oxygen consumption*. Although the latter 4 endpoints could be very interesting to estimate bioavailability influences on various candidate toxicity pathways and to calibrate dynamic energy models, we will only consider *survival* and *growth rate* in the present study. It is unclear if all experiments have been performed simultaneously, but they seem to have been treated as such in the data analysis of the original study. Cremazy et al. (2017) investigated copper effects at various Ca, Mg, pH and DOC, also over 30 days, on *survival* and *growth rate*. For the pH test series, they also measured whole body and gill Cu and Na after 24h and 30d. The pH set was reported earlier (Ng et al., 2010). They used the data to develop a conventional BLM. It is not reported in the manuscript which tests have been run simultaneously, but it seems that most tests have been run sequentially based on

the different starting weights of the fish across all tests. For the data-analysis, we will assume, however, that between-test variation in sensitivity is not significant. pH was modified and maintained by acid or base addition in both studies (not by CO_2 addition.

Before going into modelling, two possibly important points caught our attention when evaluating both studies:

1) Growth rate was more sensitive than survival in Waiwood and Beamish (1978a,b), whereas the opposite was true in the Cremazy et al. (2017) study. . It could be speculated that this could be due to the fact that different toxicity pathways are responsible for mortality vs growth, each characterised by different toxicokinetics and toxicodynamics, and that a difference in the feeding regime between both studies or the variability of these processes among different test populations (strains) leads to a different sensitivity ratio between both endpoints.
2) Cremazy et al. (2017) found that copper toxicity on survival increases with time at pH 5 and 6, but almost no mortality beyond day 5 was observed at pH 7 and above. This is in agreement with observations from Waiwood et al. (1978a,b). Thus, this appears a reproducible phenomenon. This could point to effects of pH on toxicokinetics and/or toxicodynamics of copper that govern the eventual toxicity outcome as a function of time at different pH. It could, however, also point to the fact that acidity (high H^+, low pH), which is in fact also an iono-regulatory toxicant acting on the Na balance (Morgan et al., 2000), acts jointly with copper on the same mechanism, thus explaining a longer continuation of Cu-induced mortality at low pH.

3.2.1 Juvenile rainbow trout (Cremazy et al., 2017)

3.2.1.1. pH effect

We first focus on the pH effect and we will use the 30d-LC10, LC20, LC50 values as the basis. We will also use the LC50 values at the different time points to estimate time-dependency of bioavailability relationship with pH. To avoid any interpretation difficulty

related to between-test-sensitivity (and between-lab variability in this case), we decided to only use the pH 5, 6, 8 and 8.5 treatments to estimate SpH value, since the pH 7 treatment was performed only several years later. The hardness is also somewhat different, so this would further complicate estimation. The results are depicted in Figure 4.

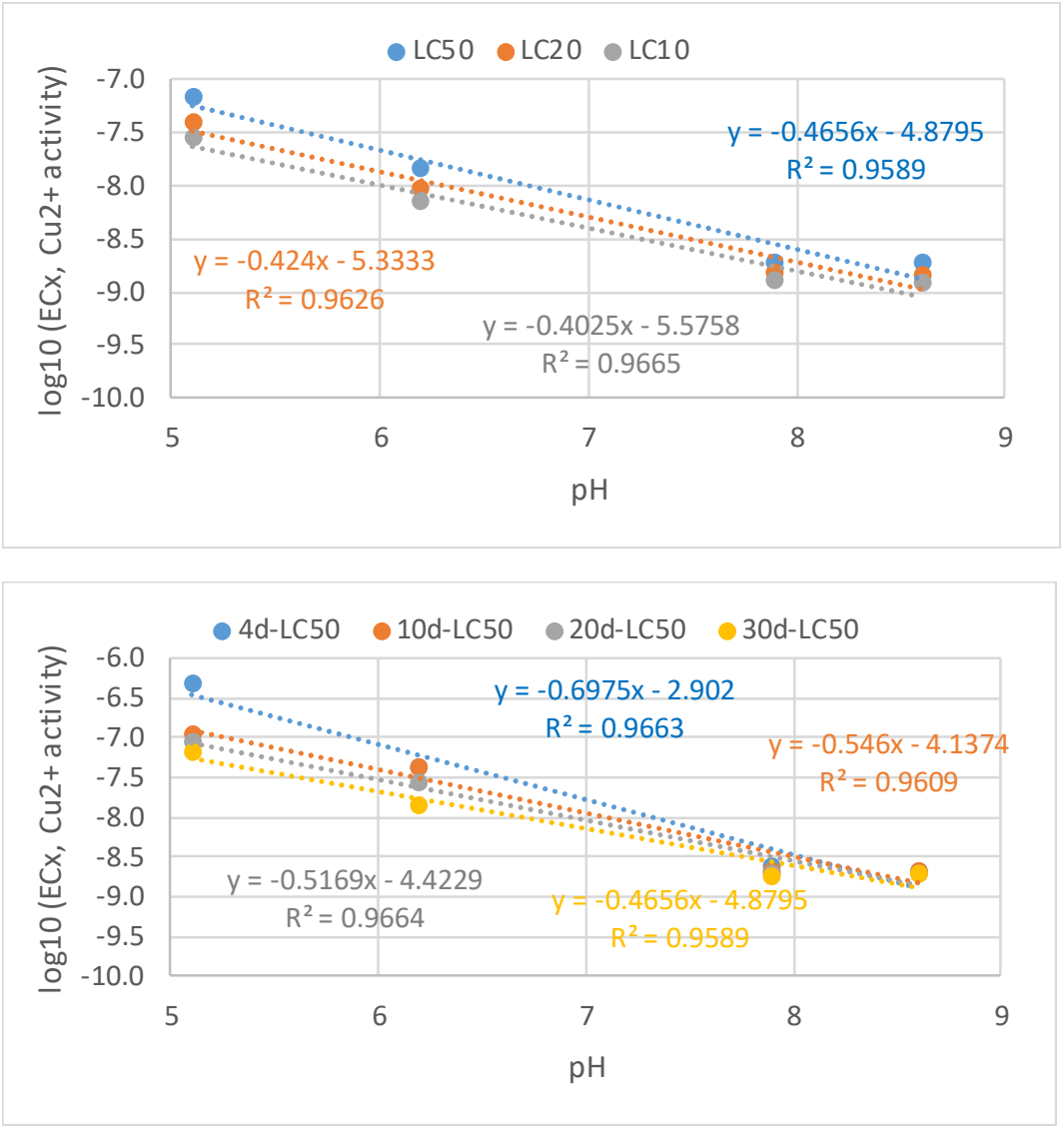

Figure 4. Effect of pH on Cu2+ ion toxicity on 30d-survival for various effect levels (top) and on LC50s for various exposure times (bottom) (Cremazy et al., 2017 data)

Considerable variation of Cu^{2+} ion toxicity on survival is observed among the different pH levels tested, i.e. up to 2 orders of magnitude. Strong log-linear correlations were observed, supporting the gBAM model structure (equation 2). S_{pH} values were similar among different effect levels (0.466-0.403), but S_{pH} decreased with exposure time, i.e. from 0.698 at 4days to 0.466 at 30 days of exposure (Table 4). The latter is in line with the observation that copper

toxicity increase with time at low pH levels and remained virtually the same beyond 4 days of exposure at high pH, as reported by Cremazy et al. (2017). In order to make our modelling exercise not overly complex, we decided to use the mean S_{pH} value of 0.431 in the final gBAM for juvenile rainbow trout (see 3.2.1.3).

3.1.2.2. Ca and Mg effects

The dataset also allowed quantifying Ca and Mg effects on copper toxicity. We found negative intercepts between 30d-LCx values and Ca^{2+} activity, which appear to be due to a lower than expected LC50 value at Ca 0.5 mM treatment. This precludes estimating a log K value. When this treatment was excluded from the estimations, this issue was solved. An additional benefit of excluding this treatment from parameter estimation was that it also resulted in the same number of treatments being considered with 10d and 20d LC50s for Ca and the same number of Mg-treamtents and the same elevated concentrations for deriving the log K_{MgBL} constant (i.e. also 1.2 and 3 mM). This increases comparability. All estimated log K values are reported in Table 4. Relatively little variation in estimated log K values values between different exposure durations and effect levels seems to exist.

Table 4. Estimates of SpH, log K_{CaBL}, log K_{MgBL}, of the gBAM for exposure durations and effect levels in juvenile rainbow trout (data from Cremazy et al., 2017).

	SpH	Log KCaBL	Log KMgBL
4d-LC50	0.698	ND	ND
10d-LC50	0.546	4.5	3.0
20d-LC50	0.517	3.9	3.0
30d-LC50	0.466	3.9	3.1
30d-LC20	0.424	4.0	3.6
30d-LC10	0.403	4.2	3.6
Final value for gBAM (mean of 30d values)	**0.431**	**4.0**	**3.4**

3.1.2.3. gBAM model for juvenile rainbow trout

Given that all gBAM parameters could be estimated directly from this dataset, this section investigates the overall predictive capacity of the gBAM. As the final value for modelling 30d-LC20 values, we selected the mean of the log K values for the three effect levels, i.e. log

K_{CaBL}=4.0 and log K_{MgBL}=*3.4*, which are identical to those derived by Cremazy et al. (2017), but somewhat higher, especially for Ca, than those derived in section 3.1.1.2 for larval fathead minnows (i.e., log K_{CaBL}=3.3 and log K_{MgBL}=*3.2*). A final value of S_{pH} = 0.431 was selected (mean of values found for the three effect levels). A K_{NaBL} is not included for now into the juvenile rainbow trout gBAM, because there were no data available on the Na effect in the Cremazy et al. (2017) study. Further on (section 4), we will argue why it could make sense to add a K_{NaBL} value to a more generic fish gBAM to increase the physiological realism.

The average intrinsic sensitivity for this dataset was calibrated using LC20 values for all test media, and a value for 30d-Q20 = -5.445 was found. The completed gBAM was then used to make predictions of observed LC20 values. We found a maximum 1.8-fold difference between observed and predicted 30d-LC20, with a geometric mean of 1.3-fold and a median of 1.2-fold. In addition, the nature and magnitude of the effects of pH, DOC, Ca and Mg (as observed in the 4 'test series') were very well reflected by the predictions (Figure 5). The observed and predicted DOC effect are almost identical, which suggests that our assumption of equilibrium speciation (despite relatively short equilibration time), combined with the 65% active fulvic assumption was appropriate for predicting the magnitude of the DOC effect on copper speciation and toxicity. The pH effect is also well captured by the gBAM, without any obvious bias. In contrast, there is some tendency for underestimation of the magnitude of the Ca and Mg effects (i.e. underestimation of toxicity at low Ca or Mg, and overestimation at high Ca or Mg). This requires some further attention as follow-up. It is not unlikely that effects of Ca and Mg are not exactly linear for the entire tested range, something that has been observed in previous metal bioavailability research, with stronger competitive effects at low than at high Mg. Possibly, here too, independent log-linear effects of Ca and Mg would yield a better match between observed and predicted toxicity, but this would require another type of gBAM.

We compared the gBAM predictive performance with that of the newly developed classic BLM of Cremazy et al. (2017), who used the same log K values for Ca and Mg competition, but also used an assumed log K_{NaBL} = 3, a log K_{HBL} = 5.8 (for H^+ competition) and included $CuOH^+$ toxicity via binding to the biotic ligand (Figure 4). Based on personal communication with the authors it appeared that they had reported incorrectly predicted 30d-LC20 values in their publication. This was due to a coding error that resulted in the non-incorporation of the K_{MgBL} in their calculations. They corrected this error and have provided us with updated, correctly predicted 30d-LC20 values (see supportive info 6.2). These values were used in the comparison. Prediction errors with their BLM were, at 1.4-fold (geometric mean), on average greater than with our gBAM. While their BLM captured the effect of Ca a bit better than our gBAM, it shows a tendency to overestimating 30d-LC20 values at pH 8. Overall, we conclude that the gBAM performs better than the Cremazy et al. (2017) BLM to explain the effects of water chemistry, notably pH, on chronic copper toxicity to juvenile rainbow trout.

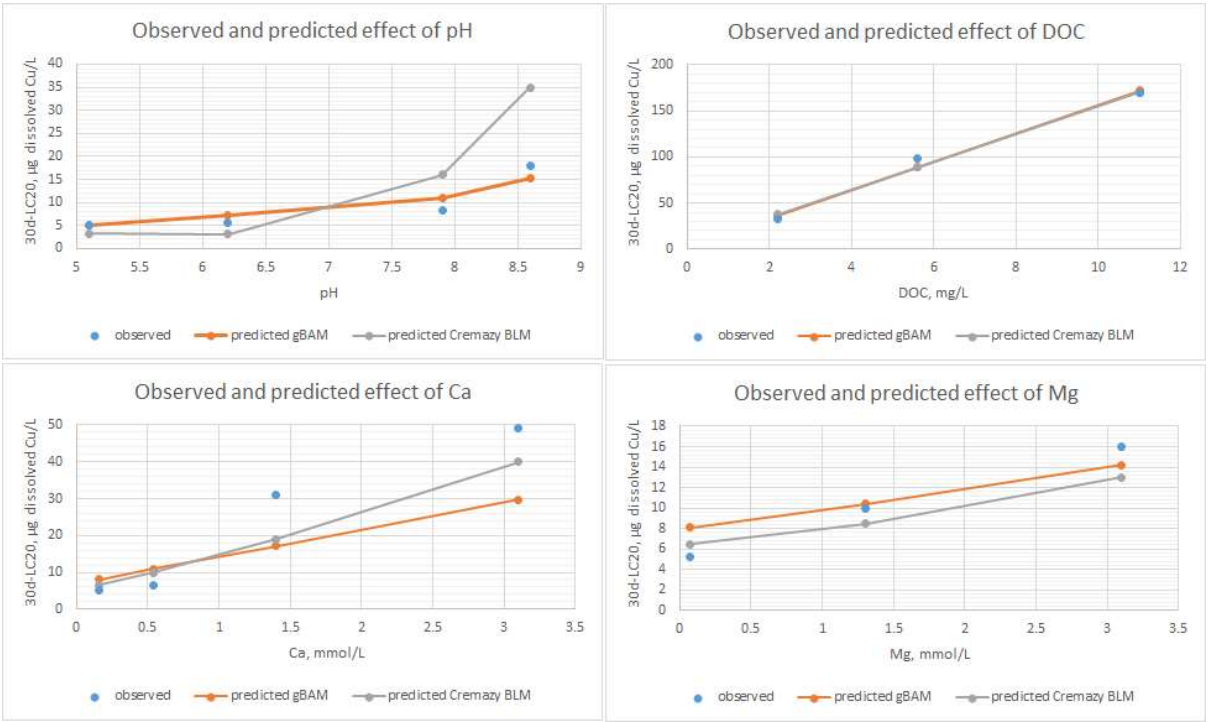

Figure 4. Predictive capacity of the chronic Cu gBAM (orange) and the Cremazy et al. (2017) BLM (grey) for juvenile rainbow trout as shown by observed (blue) vs predicted effects of pH, DOC, Ca and Mg on copper toxicity (expressed on dissolved Cu basis). Note that the gBAM and the BLM used for the predictions were developed using the observed effect data, i.e. this figure represents an auto-validation.

3.2.2 Juvenile rainbow trout (Waiwood and Beamish, 1978a,b)

We will, at this point, limit the analysis to working with the 10d-LC20 values. Future work could also make use of the growth rate data, but the limited number of copper treatments in each medium, and subsequent difficulties in estimating ECx values, would complicate this analysis. We first quantified the S_{pH} values for the three hardness levels separately, albeit each based on 2 pH levels only (low: pH 6) and high (pH 7.5-8.0). It should, however, not be forgotten that LCx values were estimated based on dose-response data with two concentrations per medium and that most LC20s were slightly extrapolated above the highest test concentrations (mostly by <15%, except 1.5-fold for pH 8, hardness 360 µg/L treatment). We found S_{pH} values of 0.274, 0.269 and 0.438 at hardness of 30, 100 and 360, respectively. Thus, relatively similar values are found, regardless of the hardness, suggesting that the effect of hardness acts independently from that of pH on Cu-induced mortality, which is in line with the gBAM formulation. The mean S_{pH} value associated with this 10d-LC20 dataset is 0.327, which is slightly lower, but relatively close to the S_{pH} value for the 10d-LC50 of 0.526 as derived from the Cremazy et al. (2017) dataset.

Log K_{CaBL} and log K_{MgBL} are impossible to determine independently, since Ca and Mg concentrations are correlated. It is also advised not to estimate them at the high pH treatments, since pH levels were correlated with hardness. However, at the low pH treatments (n=3), we can run 3 scenario's: (1) all protective effect comes from Ca, leading to a log K_{CaBL} = 3.7; (2) all protective effect comes from Mg, leading to a log K_{MgBL} = 3.7; (3) Ca and Mg have similar protective effect, leading to log K_{CaBL} = log K_{MgBL} = 3.4. All these estimates are in reasonable agreement with those derived from Cremazy et al. (2017) and with the values retained for the gBAM (i.e; log K_{CaBL} = 4.0 and log K_{MgBL} = 3.4).

As this comparison was encouraging, we tested if the 30d *survival* gBAM developed based on the Cremazy et al. (2017) data (see above) could accurately predict 10d-LC20s in the Waiwood and Beamish dataset, which showed a 13-fold variation across the six tested

media. Using a calibrated 10d-Q20 value of -4.932, to account for exposure time differences with the 30d-survival data based gBAM and possible inter-laboratory sensitivity differences, we found a maximum prediction 2.9-fold prediction error, and a median 2.1-fold prediction error (Figure 5).

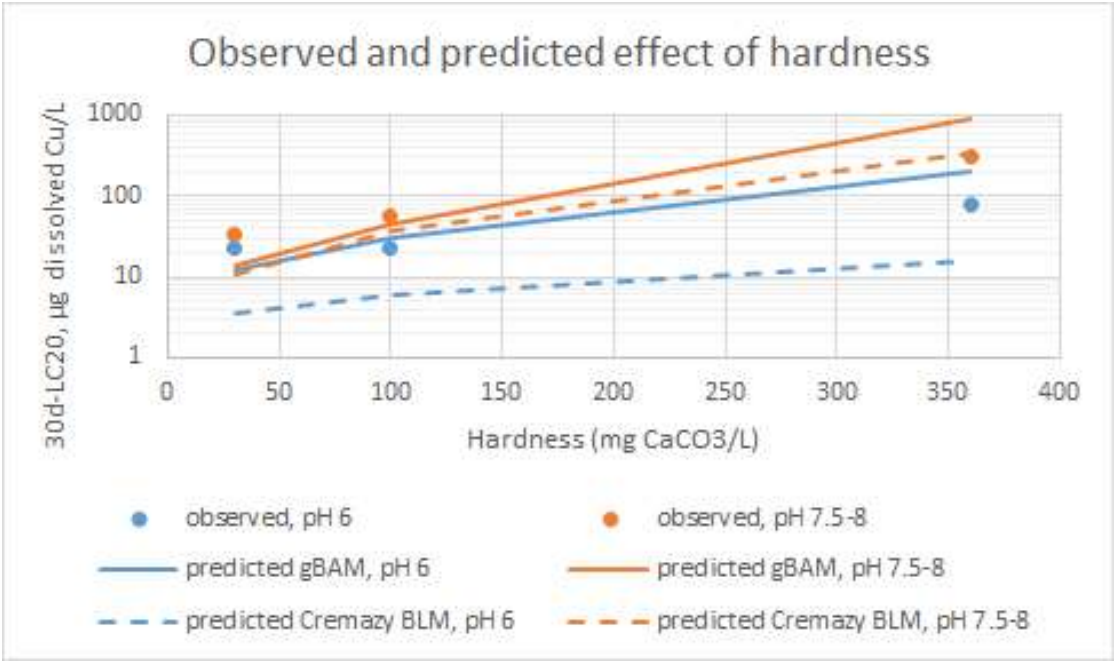

Figure 5. Predictive capacity of the chronic Cu gBAM for juvenile rainbow trout (full lines) and the Cremazy et al. (2017) BLM (dashed lines) as shown by observed (dots, Waiwdood and Beamish, 1978) vs predicted effects of "hardness" (expressed on dissolved Cu basis). Note that hardness and DOC were correlated in this experiment. Also note that this is figure represents independent validations of the gBAM and the BLM, as the observations in the plots were not used to develop these models.

Further analysis, supported by Figure 5, suggests that the magnitude of the "hardness" effect appears to be overestimated by the gBAM. It should be noted though that hardness and DOC were correlated in this dataset, and thus the apparent overestimation could also be due to an overestimation of the DOC effect, or due to the fact that the estimated DOC concentrations in the media were higher than in reality (which we cannot know because DOC was not reported).The Cremazy et al. (2017) BLM shows a similar tendency to overestimate the "hardness effect", but additionally seems to predict a larger toxicity difference between the low (blue) and high pH levels (orange) than what is observed. Thus,

here too, the gBAM seems to perform somewhat better in reflecting the observed magnitude of the pH effect.

Overall, this cross-dataset gBAM extrapolation exercise is encouraging, especially since several factors complicated the analysis and may explain the remaining uncertainty. These include the use of extrapolated LC20 values (which were derived 'by eye from log-probit paper', according to the authors, Waiwood and Beamish, 1978b), uncertainty about DOC levels (not measured, and explained in some detail above), differences in exposure time, effect level, and origin of test organisms.

Further modelling on other effect levels, exposure durations and endpoints (perhaps with specific parameter sets for each exposure duration or effect level or endpoint) would be useful to gain additional insight into the consequences of extrapolating gBAMs between different effect levels, exposure duration and endpoint). However, overall, the uncertainty about the DOC concentrations in the media of the Waiwood and Beamish studies compromise the reliability of any comparison of bioavailability relationships with studies in which measured DOC concentration data are available.

3.2.3 Conclusions, remaining uncertainties and recommendations for the juvenile rainbow trout

In any case, more weight should be given to the Cremazy et al. (2017) dataset, given that LCx estimates and speciation calculations (because availability of measured DOC values) are way more reliable than in the Waiwood and Beamish dataset (1978a,b). Yet, the latter datasets, obtained using very similar test designs, shows some interesting qualitatively comparable results with Cremazy et al. (2017), including the observed time trend of the pH effect on copper toxicity, which may point to pH dependent toxicokinetics and/or toxicodynamics of copper in juvenile rainbow trout or joint toxicity of copper and acidity. The fact that the sensitivity order between survival and growth rate was different among two

studies, however, requires further attention as it could suggest the existence of different toxicity pathways leading to mortality vs. growth effects.

Based on the Cremazy et al. (2017) dataset, an almost fully parametrised chronic gBAM (as in equation 2) for juvenile rainbow trout was developed and this gBAM accurately predicted effects of pH, DOC, Ca, and Mg. The Na effect was not investigated and hence the log K parameter for Na could not be estimated. Since Na has been shown to protect against copper toxicity in many studies, it is recommended to perform additional work enabling accurate estimation of this parameter as well for this species and life stage. It is important to emphasize that the S_{pH} parameter decreases to an important extent with exposure time, warning against unthoughtful extrapolation of gBAMs (or any other kind of bioavailability model) across different exposure durations. The gBAM accurately predicted bioavailability effects at 30d-LC20 levels.

An interesting avenue for follow-up research is the fact that the Ca and Mg effect appear to be predicted with some bias, and it was suggested that a log-linear Ca and Mg effect (as for pH) could be a better model alternative than the linear effect currently embedded in the gBAM formulation. In addition the Waiwood and Beamish (1978a,b) data also report data on energy budget related endpoints, which could be helpful in calibrating a dynamic energy model for population modelling purposes and/or to better understand mechanisms of copper toxicity.

Finally, although the chronic Cu gBAM shows great promise for juvenile rainbow trout survival, it remains to be tested (1) if the same model can also predict bioavailability effects on growth rate (e.g using the Waiwood and Beamish data, 1978a,b), and (2) if the gBAM would also perform well in field-collected waters, a typical requirement for the acceptance of bioavailability models in EU-RA.

4 REFINEMENT AND PERFORMANCE EVALUATION OF A JUVENILE RAINBOW TROUT GBAM FROM A EUROPEAN RISK ASSESSMENT PERSPECTIVE

In this section, we will explore the performance of the develop juvenile rainbow trout gBAM from a European Risk Assessment perspective. Whilst we acknowledge, based on the various analyses in section 3, that differences in bioavailability modifying effects on copper toxicity may exist between different fish species, life stages, endpoints and effect levels; it may, from a regulatory point of view be more practical to implement a single fish bioavailability model (single set of parameters) for normalising chronic fish toxicity data for copper to local or regional water chemistry. In this section, we propose to use a juvenile rainbow trout gBAM as the "single fish bioavailability model", since it is undoubtedly based on the dataset of the highest quality and with the widest range of chemistries investigated (among all available studies). After an important refinement to include a Na competitive effect, we will test how well this single juvenile fish gBAM predicts toxicity as a function of water chemistry for early life stages of fish and how far model predictions deviate from observed toxicity. With our eyes on the European Risk Assessment, we choose to perform this evaluation on the basis of LC10 and EC10 values only, since the 10% effect level is the basis for risk assessment in the EU when using chronic toxicity studies.

4.1 Refinement of the juvenile rainbow trout gBAM (including a protective Na effect)

Unfortunately, there are no data available that document the effect of Na on chronic toxicity to juvenile rainbow trout or any other fish species. However, it is very well documented that copper affects Na uptake in fish, both in early life stages as in juvenile stages, although the target of copper toxicity may shift from skin to gill during early life stage development (Zimmer et al., 2014; 2017). It is also shown that Na protects against copper effects on 7d-survival and 7d-growth of larval fathead minnow (Erickson et al., 1996) and estimated log K_{NaBL} values are in the same range as those used in biotic ligand models for acute toxicity to

fish, acute and chronic BLMs for *Daphnia magna* (De Schamphelaere et al., 2002; De Schamphelaere and Janssen, 2004) and the recent chronic gBAM for *D. magna* (Van Regenmortel et al., 2015). It is, in our opinion, reasonable to assume that the protective effect of Na on chronic copper toxicity is common to most freshwater organisms, and certainly for fish, given the ample evidence from mechanistic studies on Cu interacting with their Na homeostasis. Thus, it seems reasonable, and more likely to match with reality, to also include a value for log K_{NaBL} in the chronic copper gBAM for fish (equation 2). In the absence of data to estimate a value for chronic copper toxicity to juvenile rainbow trout, we use the same value as proposed by Cremazy et al. (2017), who took this from the original acute Cu BLM for fish (Santore et al., 2001), i.e. log K_{NaBL}= 3.0, which is also within the range of estimates for larval fathead minnow (i.e., 2.7-3.5, see Table 3, section 3.1.1.2) and *D. magna* (i.e., 2.9, De Schamphelaere and Janssen, 2004). This value of log K_{NaBL} = 3.0 was included in equation 2 (the gBAM model) and the model performance was tested at the 30d-LC10 level of the Cremazy et al. (2017) data, after calibrating the Q10-value to -5.591. The gBAM performance is shown in Figure 6 and looks nearly identical as Figure 4, which was based on the LC20 values, but which did not yet take into account a Na effect in the model. This similarity is not surprising, given that LC10/LC20 ratio's were not very different among different test media and given that the Na concentration varied only 3-fold between all test media and was sufficiently low for the gBAM not to predict any important effect of Na (i.e. (Na+) x K_{NaBL} < 1). Prediction errors were on average still equally low as for the LC20s, i.e. median 1.2-fold, geometric mean 1.3-fold. Thus, this analysis provides confidence that the addition of a Na effect in the gBAM does not affect its predictive capacity at the 30d-LC10 level. Thus the refined gBAM, with the added Na effect, as supported by mechanistic studies and toxicity studies with other species, can be used in the subsequent model-performance tests for other species and life-stages.

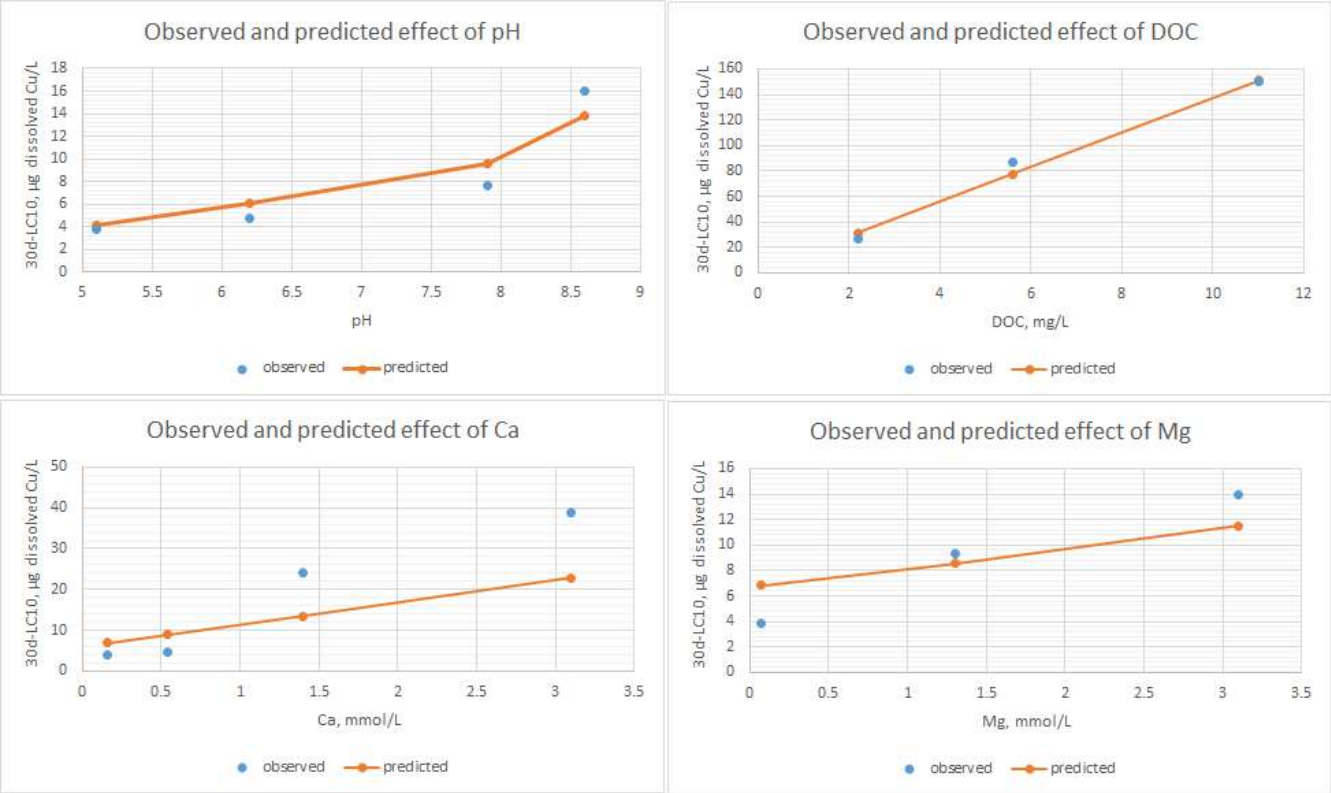

Figure 6 Predictive capacity of the refined chronic Cu gBAM (with an added Na effect) for juvenile rainbow trout as shown by observed vs predicted effects of pH, DOC, Ca and Mg on copper toxicity (expressed on dissolved Cu basis).

4.2 Performance evaluation of the refined juvenile rainbow trout gBAM (with Na effect) for early life stages of fish

4.2.1 Fathead minnow early life stages

We used the data from Erickson et al. (1996) and from OSU (2016a) in the evaluation. We used 7d-LC10 and 7d-EC60(dry wt) values from Erickson et al. (1996) and the 7d-LC10 and 7d-EC10(dry wt) values from OSU et al. (2016a). The data obtained in media with pCO2 addition to control pH in Erickson et al. (1996) were not used, because of earlier-mentioned concerns about non-natural pCO_2 increasing metal toxicity. We calibrated the refined gBAM (with Na effect) to each dataset and endpoint to account for intrinsic sensitivity differences between studies and endpoints and then made predictions of LC10 and ECx values. Figures 7 and 8 show the observed and predicted effects of pH for the Erickson et al. (1996) and OSU (2016a) studies, respectively.

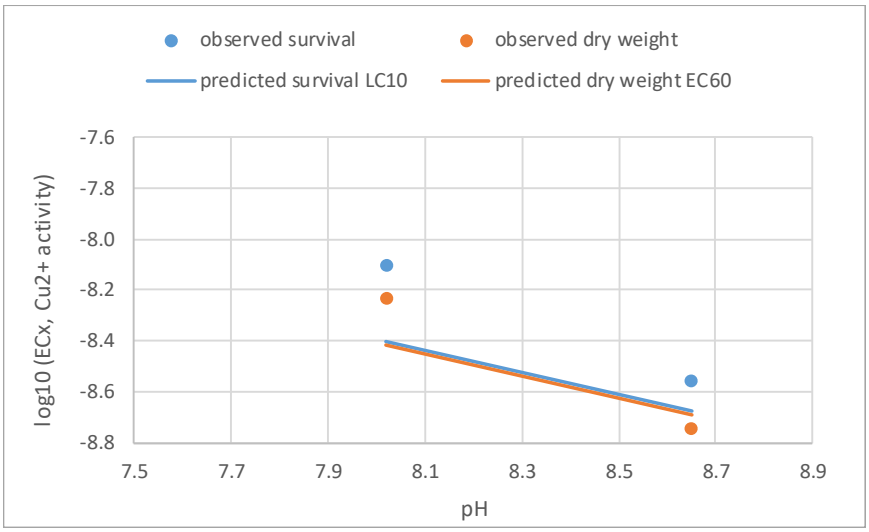

Figure 7 Observed and gBAM-predicted effect of pH on copper toxicity (expressed as free Cu2+ ion activity) to larval fathead minnow based on Erickson et al. (1996) data. This figure only shows the two highest pH levels of the study because the use of pCO2 for modifying pH to the two lower pH levels makes the data at the two lower pH levels (and hence also any comparison between observed and predicted ECx values) unreliable.

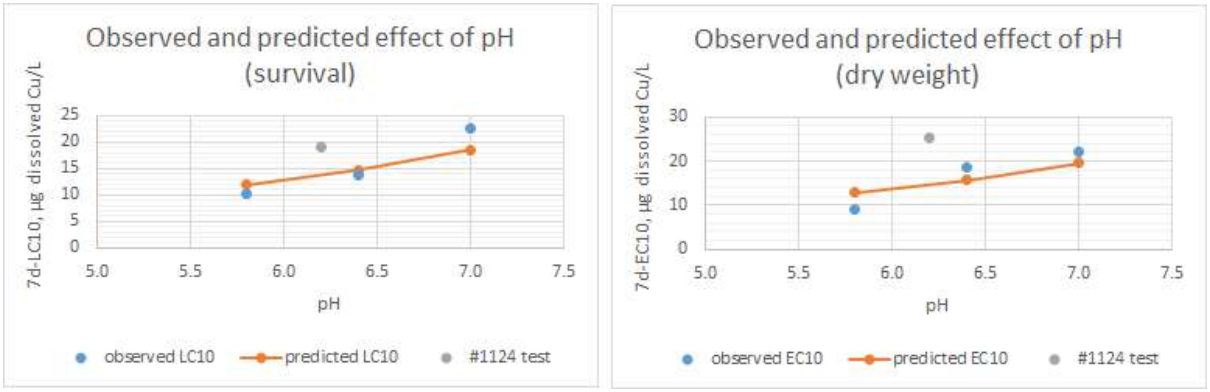

Figure 8 Observed and gBAM-predicted effect of pH on copper toxicity (expressed as dissolved copper) to larval fathead minnow based on OSU et al. (2016a, tests #1018, #1019, and #1020) for two endpoints, survival (left) and growth (right). The data from the repeated test #1024 (OSU, 2017), conducted separated in time from the other three tests are depicted for comparison.

The analysis of the Erickson et al. (1996) data shows there is a tendency for a limited underestimation of the strength of the pH effect, i.e. the slope of the observed effect appears somewhat stronger than the slope of the observed predicted (Figure 7). The observed effect of a pH change between 8.0 and 8.7 is ~3-fold, whereas the gBAM predicted effect is only ~1.9-fold. Yet, the difference between observed and predicted effect remains < 2-fold (since 3/1.9<2) (Table 3).

Table 3 Summary of the observed and gBAM predicted magnitude of the effects of separate toxicity modifying factors

Species / Study	Factor modified	Observed x-fold effect of factor on LC10	Observed x-fold effect of factor on ECx (dry wt)	gBAM predicted x-fold effect of factor	Tendency of predicted vs observed strength of the effect
Fathead minnow (Erickson et al.,1996)	pH (8.0-8.7)	2.8	3.2	1.9	Slight underestimation,<2-fold
	Ca (+2 mM)	2.4	ND	4.8	Slight overestimation,<2-fold
	Mg (+2 mM)	(4.8)	(2.0)	(1.9)	Difficult to evaluate (uncertain LCx, ECx estimates)
	Na (+2 mM)	4.2	4.8	1.5	Underestimation,>2-fold
Fathead minnow (OSU, 2016a)	pH (5.8-7.0)	2.2	2.4	1.5	Slight underestimation, <2-fold
Rainbow trout (OSU, 2016b)	pH (6.1-7.0)	(>)1.9	ND	1.1	Underestimation, difficult to quantify exactly (uncertain LC10 value at pH 7.0), but >1.7-fold

It should be noted that this evaluation is done on a free Cu^{2+} ion basis (because equilibrium speciation modelling would not work because Cu was not sufficiently equilibrated with DOM in this study). Therefore the results of this evaluation are a bit more difficult to directly relate to risk assessment practice, which is performed on a dissolved Cu basis. The analysis of the OSU (2016a) data, which are on a dissolved Cu, basis, are in this respect more straightforward to interpret from a risk assessment perspective. This analysis also shows a tendency of a slight underestimation of the strength of the pH effect between pH 5.8 and 7.0,

i.e. an observed ~2-fold effect vs a predicted ~1.5-fold effect (Table 3). However, if we also take into account the between-test temporal variability reported earlier for this study, as well as the conclusion put forward by OSU (2017), based on one more additional test, that overall, the effect of pH 6-7 on early life stage toxicity of dissolved copper to fathead minnow is not biologically significant; we conclude that the gBAM predictions (small effect of pH, i.e. ~1.5-fold) seem in good correspondence with this small or even non-significant observed pH effect (at least between pH 5.7-7.0) (Figure 8). Unfortunately, the number and range of pH levels (with reliable data) in each separate study is rather limited. In order to provide stronger support to this finding it would, however, be worthwhile to investigate in a single study the effect of pH on dissolved copper toxicity larval fathead minnows over the entire pH range in a system in which Cu is in equilibrium with the DOC and in which no pCO2 is used for pH control.

We also performed a similar evaluation for the effects of the competing cations (Table 3), based on Erickson et al. (1996). A reliable assessment of the Mg effect is difficult because of less reliable LCx and ECx estimates for the high Mg treatment. However, the gBAM shows a tendency to overestimate the strength of the Ca effect (albeit within 2-fold), and underestimate the Na effect (by >2-fold). The latter is in line with the fact that somewhat higher log K_{NaBL} values were estimated from the Erickson et al. (1996) data then the value currently included in the gBAM (based on acute juvenile fish toxicity data). Although this evaluation is based on only two data-points, it is not unlikely that there could be a biologically significant difference in the protective effect of Na (and potentially other cations) between larvae and juvenile fish, given that Na uptake shows a dominance shift from skin to gill in developing fish, which potentially results in an associated shift of the copper toxicity target (Zimmer et al., 2014; 2017). It would be worthwhile to test this hypothesis in more depth in the future and with a wider range of Na concentrations (both in larval and juvenile fish, preferably in parallel in a single study).

4.2.2 Rainbow trout early life stages

We performed the same analyses on the (limited) dataset of OSU (2016b), who investigated the effect of pH (6.1-7.0) on a number of endpoints in an embryo-larval exposure. We could only perform our analyses on the 42d-LC10 values reported for larval survival, for reasons mentioned earlier. The analysis shows that the gBAM predicts a very small effect of pH, i.e. ~1.1-fold, while the observed effect was at least ~1.9-fold (i.e., LC10 at pH 6.1 = 28.5 µg/L, LC10 at pH 7 > 54.2 µg/L, see also Table 3). This suggests a tendency of underestimation of the pH effect by the gBAM for larval rainbow trout, but as the underestimation cannot be quantified precisely because of the uncertain LC10 value at pH 7.0, this result should not be overemphasized. To our opinion, this experiment should neither be used to justify nor to invalidate the extrapolation of the juvenile rainbow trout gBAM to the larval stage.

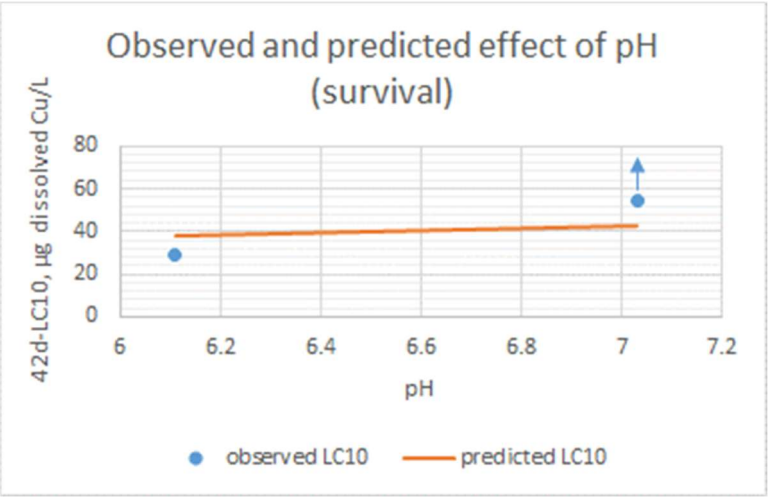

Figure 9 Observed and gBAM-predicted effect of pH on copper toxicity (expressed as dissolved copper) to larval rainbow trout based on OSU et al. (2016b). The arrow at pH 7 indicates that the true LC10 value for this medium was likely higher.

4.2.3 Conclusion on the juvenile rainbow trout gBAM performance for early life stage fish

The datasets with early life stages are not ideal to draw any strong conclusions (in either directions) on whether or not the juvenile rainbow trout BAM for chronic copper toxicity can be extrapolated to the early life stage of fish for risk assessment purposes. Thus, the results

of this model extrapolation evaluation in this section should not be overemphasized. That said, if a 2-fold error in predicting the magnitude of the effect of a toxicity modifying factor is considered acceptable from a regulatory point of view, the gBAM performs rather well in predicting the pH effect on copper toxicity to larval fathead minnow, as evidenced from data analyses on two independent studies and in two pH ranges, i.e. 5.8-7.0 and 8.0-8.7). In the low pH range (below pH 7), the gBAM predicts a small effect of pH on dissolved copper toxicity, which coincides well with the observations and conclusions drawn from a series of tests from a single lab (OSU 2016a, 2017). However, whether no definite conclusion can be drawn on how accurately the rainbow trout gBAM predicts the pH effect for rainbow trout larvae, albeit there appears to be a tendency to underestimate the effect. This requires some further attention in follow-up research. Overall, too limited experimental data is available on protective effects of major cations on copper toxicity to early life stages of fish to draw any definitive conclusions on the performance of the gBAM in this respect. Yet, when the limited data for Na (which suggests some clear underestimation of the Na effect by the gBAM) are combined with recent mechanistic insights on Na uptake in developing fish, it seems justified to consider this as a point of attention and a matter of follow-up research. Prior to embarking on follow-up bioavailability experiments and to select the life-stages or endpoints to be studied, it would be wise to determine the importance of each to the overall population-level outcomes (e.g. using population modelling).

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6 SUPPORTIVE INFORMATION

6.1 Estimated effect concentrations from Erickson et al. (1996)

Table S1 Effect concentrations for 7d-survival from test series F1 and F2 (nmol/L Cu^{2+} activity)

Test	7d-LC10	7d-LC20	7d-LC50	7d-LC60
F1-A	2.09	3.78	10.36	13.93
F1-B	5.01	9.84	31.15	43.63
F1-C	10.04	10.80	12.24	12.70
F1-D	8.88	12.32	21.57	25.41
F2-A	7.86	11.27	20.91	25.05
F2-B	2.79	3.47	5.04	5.62
F2-C	8.47	10.80	16.37	18.48
F2-D	6.17*	7.77*	11.45	12.83

*=extrapolated below lowest test concentration and therefore not considered reliable and not further used

Table S2 Effect concentrations for 7d-dry weight from test series F1 and F2 (nmol/L Cu^{2+} activity)

Test	Ed-LC10	7d-EC20	7d-EC50	7d-EC60
F1-A	0.18*	0.39*	1.45*	2.14
F1-B	<27	<27	<27	<27
F1-C	0.59*	1.10*	3.11	4.23
F1-D	0.15*	0.56*	5.33	10.28
F2-A	0.15*	0.47*	3.32*	5.86
F2-B	0.04*	0.13*	1.00*	1.81
F2-C	1.33*	2.48*	7.17	9.78
F2-D	6.03*	7.77*	11.99	13.61

*=extrapolated below lowest test concentration and therefore not considered reliable and not further used

6.2 Corrected BLM predictions of 30d-LC20s with the Cremazy et al. (2017) BLM for juvenile rainbow trout (obtained via personal communication)

Nominal conditions	30-d LC20	
	Measured	Predicted New BLM
Baseline RW	5.2 [4.3 - 6.0]	~~7.5~~ 6.5
0.5 mM Ca	6.6 [5.0 - 8.3]	10
1.2 mM Ca	31 [25 - 37]	~~18~~ 19
3.0 mM Ca	49 [29 - 69]	~~43~~ 40
1.2 mM Mg	10 [8.9 - 12]	~~5.8~~ 8.5
3.0 mM Mg	16 [12 - 19]	~~6.9~~ 13
pH 5	5.0 [1.7 - 7.3]	~~3.7~~ 3.2
pH 6	5.5 [4.5 - 6.3]	~~3.0~~ 3.1
pH 7	5.2 [4.3 - 6.0]	~~7.5~~ 6.5
pH 8	8.2 [1.9 - 9.1]	16
pH 8.5	18 [10. - 22]	~~39~~ 35
1 mg/L DOC	n.d.	-
2 mg/L DOC	33 [25 - 40]	~~37~~ 38
5 mg/L DOC	99 [75 - 120]	~~87~~ 89
10 mg/L DOC	170 [140 - 190]	170

Note: values that are ~~striked through~~ were the originally reported values